INSECT TRANSGENESIS

Methods and Applications

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1 An Introduction to the History and Methodology of Insect Gene Transfer

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1.1 HISTORICAL PERSPECTIVE ON INSECT GENE TRANSFER

The use of genetic material as recombinant DNA and the ability to integrate it into a host genome has proved to be a powerful method for genetic analysis and manipulation, providing a major new era in the field of genetics. Procaryotic gene transformation was actually realized early on, and in fact the pivotal bacterial transformation studies by Avery et al. (1944) gave definitive proof to DNA being the inherited genetic material. Continued procaryotic genetic transformation studies, indeed,

helped lay the foundation for modern molecular genetics. It is thus not surprising that geneticists attempted to duplicate this methodology in eucaryotes as well, long before eucaryotic DNA could be isolated as recombinant molecules and analyzed in a meaningful way.

The genetic transformation of insects was first attempted in *Ephestia* nearly 35 years ago, when mutant larvae were injected with wild-type DNA, with some developing into adults with wild-type wing scales (Caspari and Nawa, 1965). In subsequent studies with *Ephestia* (Nawa and Yamada, 1968) and *Bombyx mori* (Nawa et al., 1971), complementation of eye color mutations was observed after treatment with wild-type DNA. While these experiments yielded wild-type adults and at least limited non-Mendelian inheritance of the normal phenotype, it is likely that these initial insect transformations were somatic with inheritance occurring extrachromosomally. Shortly after the initial studies in moths, transformation of *Drosophila melanogaster* was similarly attempted, although delivery of wild-type DNA was achieved by soaking embryos in genomic DNA within ringers or sucrose solutions. As with the moth studies, somatic mosaics resulted, but inheritance of the reverted phenotyes was not clearly Mendelian and it was concluded that genetic transformation had occurred extrachromosomally, with episomal transmission and not chromosomal integration (Fox and Yoon, 1966; 1970; Fox et al., 1970).

More recent approaches to insect transformation began with studies in *Drosophila* that relied on the direct injection of wild-type DNA into embryos. These attempts to revert the *vermilion* (*v*) mutant line met with some success (Germeraad, 1976), although integrations were not verified beyond the genetic mapping of the complementing gene outside of the *v* locus (suggesting that a direct *v* reversion had not occurred), but the transformed lines were subsequently lost without further genetic or biochemical verification.

1.1.1 *P*-Element Transformation

Concurrent with the *vermilion* studies, the role of P factors in *Drosophila* hybrid dysgenesis was being eludicated (Kidwell et al., 1977), culminating in the identification and isolation of the *P* transposable element as the responsible agent. In now classical experiments by Rubin et al. (1982) and Rubin and Spradling (1982), *P* was first isolated from a *P*-induced mutation of *white* in *D. melanogaster,* and then developed into the first transposon-based system to transform the germline of *D. melanogaster* efficiently and stably (see Engels, 1989, for a comprehensive review of the discovery and early analysis of *P*).

P was found to be 2.9 kb in length with 31 bp inverted terminal repeats (O'Hare and Rubin, 1983), similar in general structure to *Activator*, the first transposable element to be discovered in maize by Barbara McClintock (see Federoff, 1989). Both of these elements, as well as all the subsequently discovered transposons used for insect germline transformation, belong to a general group of transposable elements known as Class II short inverted terminal repeat transposons (see Finnegan, 1989). These elements transpose via a DNA-intermediate and generally utilize a cut-and-paste mechanism that creates a duplication of the insertion site. Within the terminal repeats of these elements is a transcriptional unit that encodes a transposase molecule that acts at or near the termini to catalyze excision and transposition of the complete element. As first described by Rubin and Spradling (1982), the ability of the transposase to act in *trans* has allowed the development of binary vector-helper systems (Figure 1.1). Typically the vector plasmid includes the mobile terminal repeats of the element and requisite proximal internal sequences that surround a marker gene. The vector is made nonautonomous by having the transposase gene either deleted or disrupted by insertion of the marker gene, and thus it is unable to move by itself. The transposase is provided on a separate helper plasmid, and, after introduction into germ cell nuclei, the helper mediates transposition of the vector into the genome. The original helper was an autonomous *P* element (pπ25.1) that had the ability to integrate as well, and its presence could cause instability of the vector in subsequent generations (if not earlier). This problem was ameliorated somewhat by having much higher vector-to-helper ratios, but was solved more

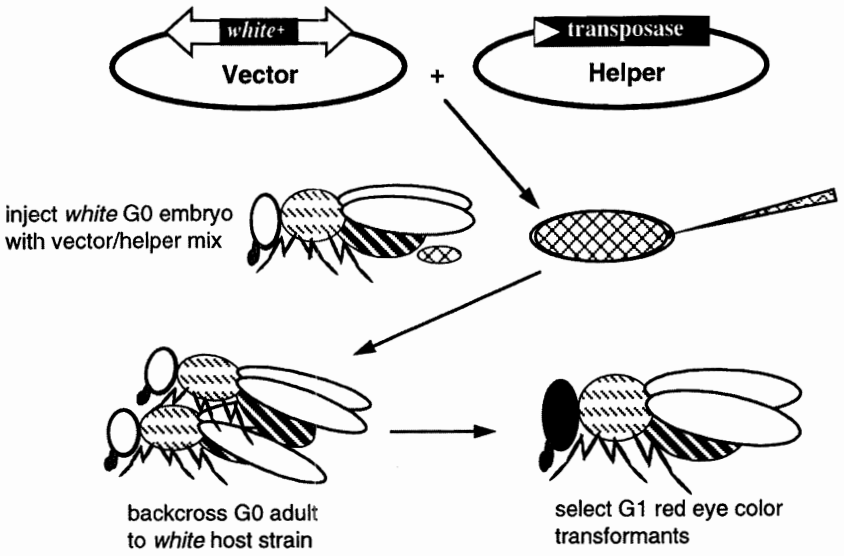

FIGURE 1.1 Schematic diagram of typical *Drosophila* transformation using a *white*$^+$ marker selection in a white eye host strain. Vector and helper plasmids are mixed at appropriate concentrations in injection buffer and microinjected into preblastoderm G0 embryos. G0 adults are backcrossed to white eye host strain flies with resulting G1 progeny screened for eye pigmentation.

definitively by the creation of a helper (pπ25.7wc) rendered immobile by deletion of most of the 3′ terminal sequence (Karess and Rubin, 1984). Without the ability to integrate, the helper plasmid is lost after subsequent cell divisions, allowing the vector to remain stably integrated.

Although the efficiency of this system relied on the high mobility properties of the *P* element, several factors were involved that allowed successful *Drosophila* transformation with this system at that time. For the *P* system in particular, the fortuitous existence of M strains devoid of *P* elements was important. These included wild and mutant strains maintained in laboratories previous to 1950, a time before *P* invaded *D. melanogaster* in the wild (Anxolabéhère et al., 1988). Most wild P strains collected after this time contain mostly defective *P* elements that provide a repressive cellular environment for *P* movement, and initial transformations in these strains would have proved frustrating, if not impossible. Curiously, some other vector systems are not, or are less repressed by host strains containing the transposon, as was found with *piggyBac* in *Bactrocera dorsalis* (A. Handler and R. Harrell, unpublished), although this may be a function of the number and structure of elements present. Given the variability of transposon regulation in any given host environment, the safest strategy for the use of a vector in an insect species for the first time is to assess its function by transient assays in the host strain before attempting transformation experiments (see Chapter 12 by Atkinson and O'Brochta).

Another important factor in the success of the *P* experiments was the ability to use a cloned wild-type gene in high molar concentrations as a transgenic marker. This is in contrast to the use of wild-type genes diluted by the rest of the genome in the original experiments. As described further on in this chapter and book, several critical factors are involved in every gene transfer system in any particular organism. These include a functional vector system, unambiguous and easily detectable markers, and simple methods for DNA delivery. All of these factors must be functional at a minimal level for successful gene transfer of any particular insect species, yet increased efficiency for any one may offset inefficiencies for the others. For example, an inefficient vector may remain useful if DNA can be delivered easily to many thousands of eggs. Obviously, optimization of all these parameters will be required for the most efficient and widespread use of the technology.

1.1.1.1 *P* Vectors and Markers

While specific differences exist between *P* and other vector systems currently used for non-drosophilid insects, optimization of these and prospective transposon and viral vectors will clearly benefit from the knowledge gained after two decades of experimentation with the *P* transposon and its use in transformation studies. Of particular importance to all researchers contemplating insect transformation is a practical knowledge of *Drosophila* transformation, and arguably it is quite worthwhile to attempt transformation of this species before any other. Presented here is a general overview of *P* transformation of *Drosophila*, and how it relates to non-drosophilid gene transfer. Several excellent in-depth reviews are available for more specific details on the structure, behavior, and use of *P*, which may be related to other vector systems, as well as transformation methodology (see Karess, 1985; Spradling, 1986; Engels, 1989; Handler and O'Brochta, 1991). Of particular interest is the book and methods manual by Ashburner (1989a,b) that review the various vectors, markers, and methodologies used for *Drosophila* transformation, as well as early techniques used to manipulate *Drosophila* embryos. We have found this information highly relevant to other insect systems.

After its description in 1982, *P* transformation was rapidly utilized by hundreds of *Drosophila* laboratories, and modifications and refinement of the *P* system occurred quickly in terms of vectors and markers (Pirrotta, 1988). This was also aided by basic investigations of transposable element structure, function, and evolution (see Berg and Howe, 1989). As noted above, the original helpers under *P* promoter regulation were autonomous elements that worked effectively, but could integrate along with the vector resulting in vector instability. A nonautonomous "wings-clipped" helper was created having its 3′ terminus deleted (Karess and Rubin, 1984), but many laboratories found it to be somewhat less effective. Subsequently, a new wings-clipped helper was created having transposase under highly active *hsp70* regulation that resulted in routine transformation (Steller and Pirrotta, 1986). It was soon realized that these frequencies, if not the ability to transform at all, was influenced by vector structure. Critical factors were vector size, placement of markers, and amounts of subterminal DNA left in the vector. Sequences within 138 bp of its 5′ end and 216 bp of its 3′ end were found necessary for *P* movement, and while the terminal repeats were identical, the adjacent sequences were not interchangeable (Mullins et al., 1989). An interesting finding was that *P* transposase has strongest binding affinity for sequences internal (~50 bp) to the inverted terminal repeats (Kaufman et al., 1989). For other vectors such as *mariner* (*Mos*1), functionality can be drastically influenced by discrete base-pair changes (Hartl et al., 1997). Vectors having requisite sequences are otherwise limited by the amount of marker DNA inserted, with the frequency of transformation inversely influenced by the size of the vector. Early tests with *P* marked with *rosy* showed that 8 kb vectors could yield transformation frequencies of 50% per fertile G_0 or greater, while the frequency with 15 kb vectors decreased to about 20% (see Spradling, 1986). Vectors as large as 20 kb could integrate, but at frequencies of 1% or less. Of course, actual frequencies depend greatly on technical ability, and various parameters including DNA purity and concentration, method of microinjection, ambient conditions, among others. We can expect other systems to be similarly affected by vector size and requisite sequences, in addition to how particular vectors and markers function in specific insect host strains.

The success of the *P* system in *Drosophila* was due in large part to the availability of easily detectable visible marker systems that rely on the complementation of a mutant allele in the host strain by a cloned wild-type gene in the vector (see Chapter 4 by Sarkar and Collins and Color Figure 1*). The original marker for "mutant-rescue" was the *rosy* gene, although this had the drawback of being cloned within a relatively large DNA fragment of ~7 to 8 kb. The *white* gene was subsequently used, but this was part of an even larger genomic fragment, and transformation frequencies were relatively low. New mini-*white* constructs (approximately 4 kb) having the large first intron deleted, with and without *hsp70* promoters, were much more effective. While these

* Color Figure 1 follows p. 108.

white markers are routinely used, they do suffer from genomic position effect variegation that acts to suppress gene expression (Hazelrigg et al., 1984; Pirrotta et al., 1985; Klemenz et al., 1987), and thus some transformants may be difficult if not impossible to detect by the visible phenotype. The same effect has been observed in tephritid fruit fly (Loukeris et al., 1995a; Handler et al., 1998) and mosquito (Coates et al., 1998; Jasinskiene et al., 1998) transformants. In one experiment comparing *white* to a green fluorescent protein (GFP) marker in *Drosophila*, we found that less than 40% of transformant G_1 flies could be detected by *white*$^+$ (pigmented eyes) expression alone (Handler and Harrell, 1999).

Additional markers based upon chemical selections or enzymatic activity were also developed for *Drosophila*, and these included alcohol dehydrogenase (*Adh*) (Goldberg et al., 1983) and dopa decarboxylase (*Ddc*) (Scholnick et al., 1983) that complemented existing mutations, and neomycin phosphotransferase (NPT or *neo*) (Steller and Pirrotta, 1985), β-galactosidase (Lis et al., 1983), organophosphorus dehydrogenase (*opd*) (Benedict et al., 1995), and dieldrin-resistance (*Rdl*) (ffrench-Constant et al., 1991) which are dominant selections not requiring preexisting mutations. These are reviewed in more detail in Ashburner (1989a) and in Chapter 6 by ffrench-Constant and Benedict. While these markers have the advantage of utilizing various mutant or wild-type host strains, and in some cases selection can be done early in G_1 development, none of them compares in the ease of use and reliability to the eye color markers. GFP markers, however, not only share the benefit of not depending on preexisting mutations, but they can actually be more effective than *white* (Handler and Harrell, 1999; see Chapter 5 by Higgs and Lewis). The primary advantage of chemical selections is that they allow selection *en masse*, which should be useful for all transgenic selections, and possibly critical for insects that cannot be transformed at a high efficiency. Now it is possible to refine chemical selections by linking the requisite genes to a GFP marker so that *bona fide* transformants can be selected and tested to increase the reliability and efficiency of the selection. Another significant benefit of GFP is that when regulated by a promoter active throughout development (e.g., polyubiquitin), transgenics can be selected early in their life cycle, saving time and resources. A caveat, however, is that expression in G_1 insects may not be detectable until late or postembryonic stages, though transgenic embryos can be detected in subsequent generations owing to a maternal contribution of GFP (see Davis et al., 1995). One drawback of using GFP markers is that a somewhat costly dedicated ultraviolet optical system is required for their detection, which may prohibit preliminary studies for many laboratories.

1.1.1.2 Use of *P* as a Genetic Tool

The *P*-element transposon has found its greatest use as a vector to import genes into the *Drosophila* genome, but has also been used (1) as a mutagenic agent to transposon-tag genes simplifying their cloning (Searles et al., 1982; Sentry and Kaiser, 1992); (2) in enhancer-trap and GAL4/UAS studies to identify and analyze temporally and spatially distinct regulatory elements (Bellen et al., 1989; Wilson et al., 1989; Smith et al., 1993; Brand et al., 1994; Gustafson and Boulianne, 1996); and (3) for targeted transposition (or site-directed gene conversion) which allows gene replacement for the creation of specific mutant phenotypes (Gloor et al., 1991; see Chapter 2 by Eggleston and Zhao). Targeting also controls for chromosomal position effects that vary when transposition is random. These methods have revolutionized genetic analysis in *Drosophila* and have the potential to expand enormously the genetic analysis and manipulation of non-drosophilid insects as well, for both basic and applied purposes. Indeed, a primary benefit of developing transposon-based vectors has been this versatility for genetic manipulation beyond simple gene transfer.

As in other systems, both transposon tagging and enhancer trapping in insects should allow the efficient identification and isolation of genes and genetic systems involved in their development, behavior, and reproduction. While a primary goal will be to use these genes or genetic elements in transgenic strains for biological control, the analysis of these genes and their use in transgenic strains for laboratory experimentation should also provide new targets for insect control using

highly specific conventional methods in addition to molecular methods. For example, a genetic dissection of neuronal pathways may reveal targets that do not exist in vertebrates, or are specific for particular insects, allowing the development of pesticides innocuous to nontargeted organisms. Genes expressed in reproductive tissues may be manipulated to produce sterility in the laboratory (e.g., for sterile insect technique (SIT); see Chapter 17 by Robinson and Franz) or their gene products may be targeted to induce sterility in the field. Manipulation of endocrine gland genes may disrupt development and reproduction, and genes expressed in antennal lobes may disrupt chemosensory reception necessary for foraging or mating. These are only a few of the potential conventional applications of information generated by basic studies of transgenic insects.

While this information will certainly improve conventional insect control strategies, the greatest potential for insect control using transgenics lies in their direct use. Many transgenic strains will be simply maintained as inbred lines; yet, some of these strains may include gene constructs that result in sterility or lethality making them difficult, if not impossible, to rear. A clever method to create strains that would normally be inviable is to utilize the yeast transcriptional activator GAL4/UAS system, which is used widely in *Drosophila* and other organisms for developmental analyses and control of gene expression (Brand et al., 1994). Essentially, GAL4 is a transcriptional activator that works by binding to upstream activating sequences (UAS) to promote transcription of downstream reading frames. It is thus possible to create a transgenic strain having a selectable or "lethal" gene linked to UAS with no negative effect in the absence of the GAL4 protein. Another strain may be created having GAL4 production regulated by a conditional, tissue-specific, or sex-specific promoter. Parental strains homozygous for the transgenes would remain unaffected, but, upon mating, their progeny will produce the UAS-linked gene product in response to the developmentally or conditionally regulated GAL4 product. For example, female-lethality may be achieved by having a strain with UAS linked to a toxin or cell-death gene mated to a strain having a female-specific promoter linked to GAL4. The UAS/GAL4 system is highly versatile as well, since libraries of GAL4 and UAS strains can be maintained with specific types of gene expression possible by mating the appropriate strains. Thus, the same UAS-cell-death gene strain used for female-lethality could be mated to a testis-promoter-GAL4 strain resulting in male sterile progeny. The use of enhancer traps and GAL4/UAS studies will help identify and test a wide array of transcriptional enhancers that can be immediately used for the sophisticated manipulation of insect populations. The power and potential of these manipulations may be vast.

1.1.2 Use of *P* for Non-Drosophilid Gene Transfer

The success with *P* transformation in *Drosophila* did not go unnoticed by those working on non-drosophilid species, and especially those interested in their genetic manipulation. The potential use of *P* in these insects was encouraged by the use of the vector to transform two drosophilid species that did not contain the *P* transposon, *D. simulans* (Scavarda and Hartl, 1984) and *D. hawaiiensis* (Brennan et al., 1984). The latter experiment was of particular interest due to the distant relationship between the Hawaiian drosophilids and *D. melanogaster*, increasing the possibility that it might function as well in non-drosophilids. Another critical factor that allowed the testing of *P* was the development of a chemical resistance marker based on the bacterial NPT gene, that allowed selection of *Drosophila* transformants by resistance to a neomycin analogue, G418 (or Geneticin®; Steller and Pirrotta, 1985). In the absence of visible marking systems, it would be otherwise impossible to select transgenics in non-drosophilid insects. Unfortunately, several years of fruitless effort ensued by several laboratories working with *P* vectors marked with NPT in mosquitoes (Miller et al., 1987; McGrane et al., 1988; Morris et al., 1989), tephritid fruit flies (McInnis et al., 1990; A. Handler and S. Gomez, unpublished), locusts (Walker, 1990), and possibly several other species, although many of these results are anecdotal due to the failure to select transposon-mediated transformants. Interesting results were obtained by several of the mosquito laboratories, however, which selected G418-resistant flies that were transformed, but apparently all occurred by low-

frequency random integrations or recombination events and not *P* transposition. These results take on added significance in light of recent results indicating transposase-dependent recombinations for other vector systems in mosquitoes (Jasinskiene et al., 1999). These observations highlight the need to consider vector function in the context of the individual cellular and genomic environment of each species.

Despite the interesting results in mosquitoes, use of the *P* vector did not result in transposon-mediated events in any of the non-drosophilid insects tested, and it appeared that neomycin-resistance selections resulted in a significant number of false positives. These and other variables involved in transformation methodology did not allow a straightforward assessment of the possible limiting factor(s). Considering vector function to be most critical, O'Brochta developed a series of *in vivo* transient excision assays that could simply and rapidly assess vector function in the insect embryo (O'Brochta and Handler, 1988; O'Brochta et al., 1991). These assays initially took advantage of *in vitro* assays for *P* function (Rio et al., 1986), and the concurrent observation of transient expression of plasmid-encoded genes injected into *Drosophila* embryos (Martin et al., 1986). These, and much more sophisticated transposition assays developed more recently (O'Brochta et al., 1994; Sarkar et al., 1997) are now used as a standard procedure to determine vector function in most insects of interest, and are discussed more fully in Chapter 12 by Atkinson and O'Brochta. The *P* excision assays quickly indicated to us that mobility of the element decreases as a function of relatedness to *D. melanogaster*, with a lack of mobility outside of the Drosophilidae (Handler et al., 1993). This provided a turning point in efforts to transform non-drosophilids, as it was realized that new vector systems were critical.

1.2 NEW TRANSFORMATION VECTORS

With the utility of *P* unlikely for gene transfer in non-drosophilids, two choices remained for vector-mediated gene transfer. One was to continue testing existing vectors or transposable elements from *Drosophila* or other organisms, or attempting to isolate new transposons or viral systems for vector development. As discussed in detail in this book, both approaches were taken with various levels of success. While *P* and other transposon-based vectors have proved their usefulness in specific species, their analysis in a variety of insects by transient assays and transformation experiments indicate that their function differs in different cellular environments, and perhaps in response to differing genomic organization. Thus, vectors successful for some species may act differently or not at all in others, and new types of vectors may be more effective for particular organisms or for particular applications. This suggests that long-term strategies for gene transfer of non-drosophilid insects will require broad-based approaches, including viral- and symbiont-based systems, as well as recombination systems and methods to improve gene targeting.

1.2.1 New Transposon Vectors

Early consideration was given to the only other transformation vector tested in *D. melanogaster*, the *hobo* element. *hobo* was discovered by its association with mutant alleles in *D. melanogaster* about the time *P* transformation was first reported (McGinnis et al., 1983; see Blackman and Gelbart, 1989), and as another short inverted terminal repeat transposon; within several years it also was developed into an efficient gene transfer system (Blackman et al., 1989). Nevertheless, there was no reason, *a priori*, to believe that the *hobo* range of function would be any greater than that of *P*. This possibility was reconsidered when amino acid sequence alignments showed that *hobo* shared related motifs with plant transposons *Ac* from maize and *Tam3* from the snapdragon (Calvi et al., 1991). Unlike *P*, whose related elements were all apparently limited to the drosophilids and closely related species (Lansman et al., 1985; Daniels and Strausbaugh, 1986; Anxolabéhère et al., 1988), *hobo* was part of a wide-ranging transposable element family. This suggested that it might have a broad range of function like *Ac* (Baker et al., 1986), and related elements might be

discovered in other species that could be used as vectors in these and related insects. Although transient assays suggested that *hobo* may have low-frequency vector function in non-drosophilids (O'Brochta et al., 1991), transformation has yet to be demonstrated for such insects. However, *hobo* has transformed *D. virilis*, which is distantly related to *D. melanogaster*, at about a 1% frequency (Lozovskaya et al., 1995; Gomez and Handler, 1997).

Importantly, homologies between *hobo* and *Ac* have allowed the identification of new elements such as *Hermes* from *Musca domestica* (Warren et al., 1994), which has been shown to be a highly active vector in *D. melanogaster* (O'Brochta et al., 1995), and it is one of the few useful vectors for non-drosophilids (Jasinskiene et al., 1998; Pinkerton et al., 2000). Notably, *Hermes* and other new insect transposon systems within the *hobo, Ac, Tam*3 (*hAT*) family (see O'Brochta and Atkinson, 1996; Handler and Gomez, 1996) have been specifically isolated for their potential use as vectors, and are discussed further in Chapter 12 by Atkinson and O'Brochta. All of the other non-drosophilid transposon vector systems were found fortuitously and were not specifically isolated for potential vector function.

Minos was the first transposon vector to successfully transform a non-drosophilid, the medfly *Ceratitis capitata* (Loukeris et al., 1995b), and it was originally discovered in *D. hydei* as part of a ribosomal RNA transcriptional unit (Franz and Savakis, 1991). Several *Minos* elements were subsequently isolated and the functional element was found to have 254-bp inverted terminal repeats and a transposase encoded by two exons separated by a 60-bp intron sequence (Franz et al., 1994). *Minos* appears to be a member of the *Tc* family, sharing more than 40% coding sequence identity with *Tc1*, and, like other *Tc* elements, it causes a TA duplication of its insertion site. The ability of *Minos* to function as a gene vector was first demonstrated in *D. melanogaster* using a *white* marked vector and a *hsp70* regulated helper (Loukeris et al., 1995a). Transformation frequencies were relatively low, in the range of about 5% per fertile G_0, although transformants were consistently produced in several experiments and *Minos* integrations were verified by Southern hybridization and sequencing of several insertion sites. Notably, chromosomal insertions were verified by PCR sequencing across the insertion sites in the parental host strain. In one of the transgenic lines, the *Minos* integration was remobilized by crossing to a transposase carrier line, that reaffirmed a *Minos*-mediated integration and provided support for the possible use of *Minos* for enhancer trapping.

The availability of the cloned *white* gene cDNA from the medfly (Zwiebel et al., 1995) made it possible to test *Minos* vector function in a medfly *white eye* host strain using *Drosophila* protocols (Loukeris et al., 1995b). Similar to the *Drosophila* experiments, several transformant lines were generated at an overall frequency of less than 5%, although this was more difficult to assess due to group matings of the G_1 flies. Nevertheless, this was the first *bona fide* transposon-mediated transformation of a non-drosophilid, and at a frequency useful for routine experiments. Although there have not been subsequent published reports of *Minos* transformation, it has been used repeatedly in the medfly (C. Savakis, personal communication), in *D. virilis* (L. Megna and T. Cline, personal communication), and *Minos* is likely to have a broad range of vector function in insects.

The *piggyBac* element was used as the second transformation agent in medflies (Handler et al., 1998) and, at the time of this writing, is the most successful vector for non-drosophilids that include dipterans, lepidopterans, and a coleopteran (although molecular verification for some is in progress). *piggybac* was originally discovered in *Trichoplusia ni* cell lines as a result of its insertion into infecting baculoviruses resulting in few polyhedra (FP) mutations (Fraser et al., 1983). Since the medfly *white* gene already had been isolated and tested as a transgenic marker in the *Minos* transformations, it was possible to test *piggyBac* transformation directly in a non-drosophilid species (Handler et al., 1998). Medfly transformation with a self-regulated transposase helper (p3E1.2 having its 5′ terminus deleted) occurred at relatively low frequencies (~2 to 5% per fertile G_0), but did indicate autonomous function for the transposon in a dipteran. Vector function was subsequently proved for *D. melanogaster* (Handler and Harrell, 1999), with frequencies elevated to 26% using a *hsp70* regulated helper, and subsequently two other tephritid fruit fly species have been transformed, *Anastrepha suspensa* (A. Handler and R. Harrell, unpublished) and *Bactrocera dorsalis* (A. Handler and S. McCombs, unpublished). A broad

range of vector function for *piggyBac* is further supported by recent transformation experiments in the silkmoth, *Bombyx mori* (Tamura et al., 2000), as well as the red flour beetle, *Tribolium castaneum* (Berghammer et al., 1999), and this work is discussed in more detail in Chapter 14 by Fraser.

The *mariner* element is another Class II transposon, originally discovered in *D. mauritiana* in association with a mutant *white* allele (Haymer and Marsh, 1986; Medhora et al., 1988). While it probably has been the most intensively studied element beyond *P*, it has taken more than a decade since its discovery for it to be used as a vector in a non-drosophilid. The *mariner* story is discussed in detail in Chapter 13 by Lampe et al., but, suffice it to say, it is part of one of the largest known transposon families traversing many orders of animals by horizontal transmission (Robertson and Lampe, 1995), and it functions accurately *in vitro* in the absence of any cofactors. Nevertheless, it has thus far demonstrated only relatively weak vector function in several drosophilids, with related active elements thus far failing to transform *D. melanogaster* after extensive testing. Only recently has *mariner* been shown to have vector function in a mosquito species (Coates et al., 1998), yet is has exhibited vector function in vertebrate species including chickens (Sherman et al., 1998) and zebrafish (Fadool et al., 1998). At present, *mariner* appears to have great potential as a broadly active vector, although it remains to be seen how useful and widely functional it will be in insects.

It is notable that the discovery of vector function for all of these transposons depended upon first developing reliable marking systems. Both of the medfly transformations depended on isolation of the medfly *white* gene cDNA (Zwiebel et al., 1995), and the testing of *Hermes* and *mariner* in *Aedes aegypti* relied on the finding that the *D. melanogaster cinnabar* gene could complement a mosquito white-eye mutation (Cornel et al., 1997). Thus, while much effort and interest have been focused on vector systems (see O'Brochta and Atkinson, 1996), the lack of suitable transgenic marker systems has been an equal, if not greater, bottleneck for successful insect transformation.

1.2.2 Viral and Symbiont Vectors

While transposon-based vectors are preferable for the creation of stable transgenic strains at this time, and especially for basic studies of gene expression and genetic manipulation, it is realized that different types of systems will be necessary for particular field or experimental applications, and for particular insect species not amenable to transposon function or the typical methodology used for germline transformation. For example, many insect species have long generation times or complex life cycles that make untenable the testing of germline transformation, or the creation of mass-reared transgenic strains. For some purposes, the need to create stable transgenic strains is not essential, and the development of extrachromosomal transient expression systems that might be much simpler to create, could be a higher priority if not preferable. Such transient systems might have the added benefit of acting as carriers for inefficient transposon vectors or gene-targeting systems allowing them to perdure within cells, increasing their chance for integration. Thus, while the development of germline transformation systems for non-drosophilid insects has been a priority, the need for other types of systems for particular purposes and particular species is clearly of equal importance. For these situations infectious agents such as viruses or gene expression from endosymbionts may be more efficacious, and several of these systems are discussed in this book.

Several viral systems are under consideration such as the Sindbis RNA virus that is highly effective as an expression system both *in vitro* and *in vivo* (see Chapter 9 by Olson), and DNA viruses such as densoviruses that have been used as transduction agents in mosquito larvae (see Chapter 8 by Carlson et al.). Retroviruses have long been used as vehicles for gene transfer into mammalian cells (Eglitis and Anderson, 1988), and recently the host range of a mammalian retroviral vector was increased considerably by pseudotyping to include vertebrate and invertebrate systems including several insect species (see Chapter 7 by Burns). With the recent elucidation of retroviruses in insects such as *gypsy*, these systems also have potential for insect vector function with minimal modification (see Chapter 10 by Terzian et al.).

A bit different from the typical concept of germline or somatic (transient) transformation is the use of symbiotic organisms to express genes of choice in a host organism, a type of gene transfer referred to as paratransgenesis. Several systems are under development that have great potential for particular applications. For example, several prospective field applications will require driving transgenes into an insect population and bacterial endosymbionts, such as *Wolbachia,* that can spread through populations very rapidly have considerable potential for achieving this (see Chapter 15 by Sinkins and O'Neill). Of course, the genes to be spread must be transferred into the endosymbiont first, and this may take advantage of viral or transposon vectors. Bacterial symbionts of *Rhodnius prolixus* have already been transformed with genes lethal to parasitic trypanosomes that cohabit in the insect host, and the use of such paratransgenic insects for disease prevention may be implemented shortly (see Chapter 16 by Beard et al.).

As the development of more sophisticated transgenic strains becomes necessary for applied uses, we will find the need for gene transfer of multiple transgenes that are highly stable and noninteracting. The greatest assurance for having a widely applicable genetic toolbox for many different species will only come from the development of vector systems that are mechanistically distinct and varied.

1.3 TRANSFORMATION METHODOLOGY

Since *Drosophila* is the only routinely transformed insect, it is the best model system for transformation methodology, although, undoubtedly modifications will be necessary if not critical for other types of vectors and insect systems. Our studies with tephritid fruit flies have generally followed the standard *Drosophila* procedures (see Spradling, 1986), and most other studies with other insects have done so as well. Although several of these studies have been successful, it is quite possible that modifications in injection buffer, DNA concentrations, and DNA delivery might greatly improve gene transfer efficiency. Some specific modifications developed for mosquito transformation are discussed by Morris (1997). While primary concerns for non-drosophilid transformation usually centered on functional vectors and selectable marker systems, the ability to deliver DNA into the germline or soma of the host organism has been equally daunting, and, at present, it is probably one of the greatest general roadblocks.

1.3.1 DNA Preparation

The preparation and amount of DNA injected is critical to successful transformation. Early *Drosophila* experiments utilized only cesium chloride purified plasmid DNA, usually requiring double ultracentrifugation with rigorous removal of ethidium bromide and any organic solvents (see Sambrook et al., 1989). The recent availability of plasmid preparation kits provides much simpler methods. While we and others have successfully transformed with plasmid prepared with such kits, for some plasmids grown in some particular bacterial hosts, contaminating toxic proteins may not be easily removed and the cesium method is generally the most fool-proof. Qiagen now provides an endotoxin-free kit that may alleviate some of these concerns (see Appendix to this chapter for location and contact information for this and other companies mentioned).

Plasmid DNA for injection is usually a mixture of the desired amounts of vector and helper (or vector alone) that is ethanol precipitated and resuspended in injection buffer. Most insect transformations have used the *Drosophila* injection buffer (5 m*M* KCl, 0.1 *M* sodium phosphate pH 6.8), although it is likely that this is not ideal for all insects. Resuspended DNA should be used within a few days, and kept frozen until use, with an aliquot run on an agarose gel to ensure DNA integrity and the general concentration desired. Previous to injection, the DNA should be centrifuged at 12,000 × *1212* for 5 min to eliminate any particles that might clog needles or contaminate the eggs.

Generally, total DNA concentrations have not exceeded 1 mg/ml with vector/ helper ratios ranging from 2:1 to 9:1, with actual molar ratios being a consideration. Excessive amounts of DNA are considered to be harmful if not lethal to embryos, probably because of the nucleic acids themselves and unavoidable contaminants in the solution. It is likely, however, that many embryos larger than *Drosophila* can withstand higher concentrations or amounts injected. Other considerations are the size of the plasmid (usually the vector), since as plasmid size increases the number of molecules injected will decrease for a standard DNA concentration, but increasing the concentration may result in shearing the molecules during injection. Shearing may be alleviated by larger-bore needles, although embryo survival may be compromised. These and other trade-offs are pervasive in gene transfer methodology, and the best system for any particular insect must be determined empirically by extensive control experiments.

1.3.2 Dechorionation

Drosophila benefits from having an easily removable chorion, either manually or chemically, and can be quickly desiccated for microinjection of DNA. Manual dechorionation is perfomed by gently rolling freshly laid eggs on double-stick tape with forceps, with the egg generally popping out from the chorion. Chemical dechorionation uses a diluted hypochlorite solution (liquid bleach) at a final concentration of 1.5 to 2.0% (note that hypochlorite concentrations in bleach vary around the world). The bleach should also be fresh — open for no more than 2 to 3 weeks, with diluted solutions being no more than 2 days old. The time and concentration of hypochlorite treatment must be determined empirically. If a 1.5% solution does not dechorionate within 2 to 3 min, slightly higher concentrations should be tried. After dechorionation, eggs must be repeatedly washed in distilled water, which is usually aided by the addition of Triton-X 100 or NP-40 (0.02% final concentration). After three washes the eggs can be prepared for desiccation.

Depending on the number of eggs collected, hypochlorite dechorionation can be done in a watch glass or small culture dish using a drawn out Pasteur pipette or syringe to remove fluid and a brush to swirl and move the eggs. Larger numbers of eggs are more easily (and quickly) manipulated by using a small Buchner funnel (~45 mm inner diameter) and a filtering flask. Eggs collected (or washed) onto white filter paper are placed in the funnel and rinsed in water which is gently drawn off with a water vacuum (use very low vacuum and control by keeping flask stopper loose). Diluted bleach is added to the funnel, swirled around, and gently drawn off after the apppropriate time. Wash solution is quickly added with a squirt wash bottle, swirled, and drawn off. The filter paper with eggs is taken out of the funnel, and the eggs washed onto black filter paper (eggs are more easily observed on black filter paper, which is bleached by the hypochlorite). Washing is repeated two to three times with very gentle aspiration (filter should always be wet and not sucked dry or eggs will be crushed).

1.3.3 Preparation for Embryo Injection

Dechorionated *Drosophila* eggs are kept moist on damp filter paper (from the last wash) and their development may be slowed by keeping them in a cool incubator or ice bucket (although not directly on ice). By using a fine brush (000) or forceps, eggs are placed on a thin strip (1 to 2 mm) of double-stick tape placed on a slide, often at the edge. We have found it more convenient to have the tape on a rectangular coverslip (30 × 22 mm) which is then placed on top of a slide on the mechanical stage. *Drosophila* eggs are typically injected under halocarbon oil, although recently some laboratories have found it possible to inject in open air, using food coloring in the DNA mixture to visualize better the injected DNA. We continue to inject under oil, and therefore place the tape inside a thick rectangle drawn with a wax pencil which holds the oil around the eggs (we can fit two rectangles with tape on each coverslip; wax lines should be ~5 mm from the edge of

the tape). Eggs are placed on the tape with with the posterior end facing the edge of the coverslip so that injection may be made near the pole plasm.

Most dipteran eggs we have injected must be desiccated to reduce the internal volume and pressure so that injected DNA can be accepted without extrusion. Proper desiccation may be the most critical factor for good injection and embryo survival, and optimal desiccation times must be determined empirically for each experiment, and often change within a several-hour period. Over-desiccation often results in death, and underdesiccation often results in extrusion of the DNA and yolk, resulting in death, sterility, or a lower gene transfer frequency for those that survive. Although embryos can sometimes survive considerable loss of material, these embryos are likely to be sterile due to loss of pole plasm, or nontransformed due to loss of injected DNA. We typically desiccate in open air at 22°C and 50% relative humidity or lower for 8 to 12 min. Desiccation times are certainly affected by ambient temperature and humidity, and may be better controlled by placing the eggs in a closed chamber with a drying agent, with or without a gentle vacuum. Obviously manual dechorionation requires less desiccation time than bleach dechorionation.

1.3.3.1 Needles

Close in importance to proper desiccation for microinjection are properly prepared needles. To a certain extent there is a trade-off between desiccation and needles in that very fine sharp needles can inject well into underdesiccated eggs, with the benefit of better viability. On the other hand, large-bore needles do not clog as easily and may be necessary to prevent shearing of large plasmids, but eggs must be desiccated as much as possible to accept the injection. For insects such as moth and mosquito species where dechorionation is not possible, large-bore needles are required to "cut through" the chorion and not get clogged. Typically, needles are drawn out on needle pullers usually used for creating glass electrodes for neurophysiology and can be found at World Precision Instruments (WPI) and Narishige, among other suppliers. The same glass capillaries for electrodes may be used (some including microfilaments allowing easy fluid flow) or more simple micropipettes (such as 25 µl Drummond microcaps). Previous to drawing out the needle, the capillaries should be siliconized or silanized by one of a variety of methods (e.g., Sigmacote from Sigma), and thoroughly washed and dried. For embryos that have a soft chorion or can be dechorionated, a finely drawn-out needle with the tip scraped off along the edge of the glass slide (that supports the coverslip) before injection works quite well. For the many embryos that cannot be dechorionated, such as mosquitoes (see Jasinskiene et al., 1998), beveling the tips of the needles will probably be required, and this is helpful for all injections and especially those requiring large-bore needles (Morris, 1997). We use a beveler available from Sutter Instruments (BV-10), and WPI and Narishige also provide similar types of needle bevelers.

DNA may be backfilled into the needle or sucked up prior to injection. Backfilling may result in less shearing, and needles for backfilling can be created from siliconized 100 µl microcaps that are drawn out over a flame and broken in the middle. A few microliters of DNA sucked into these needles by capillarity can then be backfilled into several injection needles.

1.3.4 Microinjection Apparatus and Procedures

The typical apparatus for *Drosophila* microinjection requires an inverted or stereozoom microscope having a 10 to 80× magnification range and transmitted light, a mechanical stage, micromanipulator, and a mechanism for DNA injection (Figure 1.2). Direct illumination (instead of or in addition to transmitted) is required for embryos that are pigmented or cannot be dechorionated. The general procedure involves aligning the needle at the injection site with the micromanipulator, while the actual injection is done by using the mechanical stage to move the egg into the needle. Once inside the egg, DNA is injected manually or with an electronic air-pulse system. *Drosophila* is typically injected under oil, although early injection experiments were done in open air, and several methods

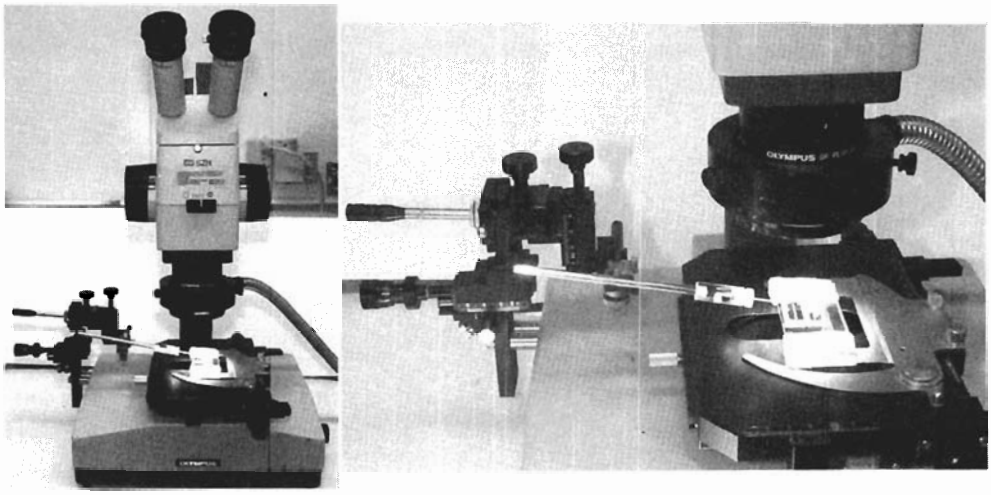

FIGURE 1.2 Microscope setup for insect embryo injections. The setup includes an Olympus SZH stereozoom microscope with a transmitted light base and BH2-SH mechanical stage (with SZH-STAD1 adaptor). A Narishige MN-151 micromanipulator with a B-8B ball joint pipette holder is mounted on the microscope base, with an attached WPI MPH-1 micropipette holder. The air-injection system is not shown, but it connects perpendicularly into a female luer fitting within the plastic cylinder portion of the needle holder. Embryos prepared for injection on a coverslip are placed on a slide taped to a U-shaped plastic carrier for elevation.

were used to seal the puncture wound including a dissolved gum damar and a 1.2% sulfosalysilic acid solution (see Ashburner, 1989a). While *Drosophila* is again injected in open air by some laboratories, typically the injection site is not sealed, although for other insect embryos that cannot be submerged in oil and require large-bore needles, sealing the puncture should limit leakage and increase survival. An alternative sealant is any one of a number of "super" or "crazy" glues, although the effect on viability of any of these sealants should be tested on uninjected and injected eggs. We continue to inject several dipteran species under oil without sealing.

Drosophila injections were originally performed with an inverted microscope because they had or could be fitted with a mechanical stage, there was working space for a micromanipulator, and, in all likelihood, they were generally available. We found more recently that the Olympus SZH stereozoom microscope (now SZX) with a transmitted light base could be fitted with a flat Olympus stage (model BH2-SH) which is almost ideal for easy handling. The mechanical stage and adaptor (SZH-STAD1) can actually be fitted on most stereozoom dissecting microscopes from other manufacturers after tapping screw holes for the adaptor into the base; however, a longer carrier pole for the microscope body may be needed for the additional height (400 mm pole used on Olympus). This setup has the advantage of zoom optics and easy handling of eggs and needle while under view, and for non-tissue-culture laboratories, the scope has utility when the injection apparatus is removed (e.g., scoring transformants).

The micromanipulator should be adjustable in three dimensions (*X*, *Y*, and *Z* axes), and finely adjustable in the axis used for proper needle height (axis can vary depending on how the manipulator is mounted). We use two manipulators, which work well: a Leitz manipulator that is free-standing (current Leica manipulators may differ) and a Narishige MN-151 manipulator (with B-8B ball joint pipette holder) that can be mounted with adaptors onto the microscope base. The Leitz manipulator was originally suggested for *Drosophila* injections, but we prefer the Narishige for its simplicity and it is considerably less expensive (and the lower cost models MN-152 and 153 also may be used).

Apparatus for DNA delivery varies greatly — from simple connections using a 50-cent syringe and tubing, to air-pulse controller systems costing up to $20,000. As might be expected, the cheaper

apparatus probably works best for those who routinely inject and have a refined technique. We use something in between which is relatively inexpensive and efficient, and modest upgrades are also possible that mimic the very elegant and expensive systems. Our system is also based on components used in neurophysiology that include a pressurized air-tank or house air-supply connected to high-pressure tubing with an in-line pressure gauge and regulator, and an electric valve operated by a simple switch and battery supply. These components are available from Clippard who sells through regional suppliers, at a total cost of less than $120. A male luer fitting or cutoff 1-ml luer syringe is fitted at the end, and this connects into a WPI MPH-1 microneedle holder. The switch and battery holder are available from any local electronics store. We find this system to be quite inexpensive, easy to use, and efficient. An upgraded air-pulse system is the pneumatic picopump from WPI (PV830), which has pressure and vacuum controls and a foot switch for approximately $2000. The vacuum capability allows the uptake of DNA through the needle and is useful in unclogging needles. Several laboratories use a somewhat more elegant version of the picopump from Eppendorf, currently marketed as the Transjector 5246 (that can be used with their micromanipulator called InjectMan), which is quite expensive. Both WPI and Eppendorf sell premade microneedles, but these seem prohibitively expensive ($3 to $5 per needle) for routine injection experiments where many needles may be required.

1.3.5 Methods for DNA Delivery

Although microinjection is currently the method of choice to deliver large quantities of DNA into embryos, as noted above, different methods of DNA delivery have been tried ever since insect gene transfer experiments were first attempted. Some of the first were variations on soaking embryos in DNA, that attempted to increase efficiency of DNA entry by trying first to permeabilize the eggs with organic solvents (Limbourg and Zalokar, 1973). The more recent development of methodologies and equipment for introducing DNA into plants, cell lines, and bacteria have offered a wide range of possibilities. The most well known of these include lipofectin reagent, biolistics (originally ballistics), electroporation, and modifications of microinjection. Most of these have been attempted with *Drosophila* or other insects for at least transient DNA delivery and expression, and while none have proved efficient yet for routine germline gene transfer, further experimentation is certainly warranted for those species not amenable to efficient microinjection. Certainly for those systems that allow DNA delivery *en masse*, routine gene transfer may be possible even with high mortality and inefficient vector systems. Modifications of microinjection also have been attempted, such as injection into ovarian egg follicles previous to oviposition (Presnail and Hoy, 1992), or injection into the female hemocoel for DNA uptake into egg follicles along with vitellin. For some of these methods, information has not been published, is found in abstracts, or is anecdotal. The following brief overview should provide some useful information that may be used as a basis for further experimentation.

1.3.5.1 Lipofection

The development of cationic lipids that form liposomes encapsulating DNA has provided a routine method for DNA delivery into cultured cells, that occurs after liposome fusion with cell membranes. Modified forms of the cationic lipid have been developed and are commercially available for the procedure known as lipofection. Although used routinely for transfection of cell lines, lipofection also has allowed DNA delivery into cells *in vivo* in a variety of animal systems (Nicolau et al., 1983; Felgner et al., 1987), and transient expression has been reported for cultured mosquito salivary glands (Morris et al., 1995) and heart tissue from the Pacific oyster (Boulo et al., 1996). Possible advantages of using lipofectin would be the uptake of DNA into egg follicles from maternal hemolymph, or the uptake of DNA into the germline after pole cell formation, allowing a considerably longer time for injection during blastoderm formation. A systematic analysis of lipfectin–DNA mixtures with functional vectors in *Drosophila* or other species should be a high priority.

1.3.5.2 Biolistics

Two DNA delivery systems using mechanical means are biolistics and electroporation. Biolistics involves the coating of microscopic pellets (usually gold or tungsten microparticles) with DNA by ethanol precipitation, which are bombarded into cells or tissues. It was derived from a ballistic method, so-called since it actually used a modified form of a shotgun to shoot the particles into plant tissue (Klein et al., 1987). A highly interesting study used the ballistic method to deliver DNA into *Drosophila* embryos successfully, resulting in high frequencies of transient expression, and in a *P* transformation experiment a single transformant from several thousand eggs was recovered and verified (Baldarelli and Lengyel, 1990). Although this report met with great optimism, repetition of the experiment has not been reported. Ballistics was subsequently modified to a biolistics method using pressurized helium within a chamber and is commercially available from BioRad. Biolistics is routinely used for plant transformation, and a more recent modified apparatus is the Helios Gene Gun System® that can be used for subcutaneous injection. Further details on these current systems are available from the company (see catalog and Web site). An apparent problem using the biolistics PDS-1000/He chamber for insects has been disintegration or dipsersal of eggs after the high-pressure bombardment. Miahle and Miller (1994) addressed this problem and successfully achieved transient expression in mosquito eggs by having the DNA-coated particles in an aqueous suspension. They increased levels of reporter gene expression (luciferase) significantly using this suspension under high or low pressure, although transformation has not been reported. These results indicate that a ballistic or biolistic method can effectively deliver DNA into insect embryos and, at some level, achieve transformation.

1.3.5.3 Electroporation

DNA delivery by electroporation has also received considerable attention in recent years. Essentially, cells in solution are given an electric shock which serves to increase pore size in cell membranes allowing the passive introduction of molecules from the solution. It is used routinely for bacterial transformations, and modifications in the shock parameters, including waveform, resistance, and voltage, have optimized DNA delivery for a variety of procaryotic and eucaryotic systems. Multicellular organisms have been more challenging, although great interest was generated by the relatively high frequency of xanthine dehydrogenase transient expression in *Drosophila* larvae that had been electroporated as embryos under fairly standard conditions (Kamdar et al., 1992). Additional challenges exist for insect eggs that exceed the size of, or cannot be submerged in, standard cuvettes (usually 1 to 4 mm wide). Leopold and colleagues (1996) have successfully electroporated eggs under uniform conditions from lepidopteran and dipteran species using a slot cuvette design with electrodes placed on a microscope slide. While the reports for successful DNA delivery, as concluded from transient reporter gene expression, are highly encouraging, germline transformation in *Drosophila* has not been reported. Nevertheless, the benefits of such a procedure and success with transient expression from these initial reports indicate that continued high-priority efforts in this area of DNA delivery are also warranted.

Outlined above are the primary systems of DNA delivery currently being used or considered, although the range of size, structure, and habitat of insect eggs suggests that any system successful for a particular species (or related species) will require modifications for others. Microinjection of oviposited eggs has already been modified so that DNA may be delivered *in situ* in mites and wasps (Presnail and Hoy, 1992; see Chapter 19 by Hoy). Preliminary experiments in our laboratory indicate that DNA injected into female abdomens is taken up by maturing egg follicles and can be recovered in oviposited eggs, although recovered plasmid amounts have been low (<100 molecules per egg) (S. Gomez and A. Handler, unpublished). Another variation on injection involves the puncturing of eggs with a tungsten needle coated with DNA, and a combination of methods such as the use of lipofectin-reagent with any of the various mechanical methods may help increase their efficiency.

Continued testing for all these methods is clearly warranted now given the new vectors and markers that have become available since most of the methods were originally considered.

1.4 IDENTIFICATION OF TRANSGENIC INSECTS AND TRANSGENES

A significant portion of this book is dedicated to markers for transgenic selection. These markers are gene products that restore a normal phenotype in a mutant host, confer a new visible phenotype, or confer resistance to a chemical or drug. Some, and possibly most of these markers, provide reasonably convincing evidence that a marked G_1 insect is indeed a transformant. Yet, there are a variety of reasons to remain cautious, and it is now realized that transgenics must be identified unambiguously by definitive molecular tests. This is especially so for a proof of gene transfer in an insect species with a new vector system for the first time. For "mutant-rescue" selections resulting in a wild-type phenotype, nontransformants may be erroneously selected due to strain contamination and, more rarely, reversions of the mutation. As noted above for G418 selections, non-vector-related chemical resistant organisms may be selected, and this may occur in the host strain itself, or in host strain symbionts. Beyond the simple determination of gene transfer, an assessment of vector activity and the potential release of a transgenic strain depend on knowing the number and possibly the chromosomal location of the vector integrations. In addition, an assessment of vector stability and the potential use of a vector as a genetic tool depends on knowing whether an integration was vector mediated, as opposed to a fortuitous or illegitimate recombination. Non-vector-mediated integrations cannot, typically, be remobilized, which may add to their stability, although this might hinder the use of the vector for enhancer-trap studies. Finally, there may be safety issues or practical concerns related to exactly what part of the vector entered the host genome. Most vector plasmids have at least one or more antibiotic resistance genes, bacterial reporter genes, and an origin of replication. If vector integration occurs by recombination and not transposon or viral transposition, then extraneous DNA may inadvertently enter the genome as well. It should be noted that *P* vector insertions, which normally integrate individually, have been detected as multimeric concatamers. This and other types of rearrangements may occur previous to integration, or afterward if the vector transposase or cross-mobilizing system exists in the genome.

The determination of a chromosomal integration, and the number of integrations (if fewer than 10), is most easily achieved by Southern DNA hybridization; however, extrachromosomal DNA (e.g., nonintegrated injected plasmid) may be detected if in large enough quantities. Nevertheless, proper diagnostics with carefully chosen restriction site digestions and probes should normally yield unambiguous results (see Sambrook et al., 1989). Some studies have utilized direct PCR for molecular detection, but contamination may be problematic since vector plasmids are probably widespread within the same laboratory, and at the very least control reactions are necessary using several primer sets to plasmid sequence outside of the transposon (although this will detect non-integrated plasmid as well). Chromosomal integrations and their number can also be determined by *in situ* hybridization to chromosomes (see Ashburner 1989a, b), and *Drosophila* transformants have been typically analyzed in this way, but this is most easily performed in the relatively few species with large polytenized chromosomes.

The most definitive proof of vector-mediated chromosome insertions is by sequencing the insertion site junctions, since most transposons create an insertion site duplication that should be diagnostic for that vector. Sequencing an insertion site is most simply achieved by isolating the junction sequences by inverse PCR (Ochman et al., 1988), but this is most straightforward for genomes having a single integration, and, again, plasmid contamination must be avoided. For multiple integrations within a transgenic strain, it may be necessary to create and screen small genomic libraries, and this was found necessary for mosquito strains where only one of the junction fragments could be isolated by inverse PCR (Jasinskiene et al., 1999). An alternative strategy useful

in *Drosophila* is plasmid rescue (Steller and Pirrotta, 1986), which is based on having a pUC vector backbone within the transposon vector termini. Thus, transformed genomic DNA can be digested, religated to form circles, and transformed into bacteria. Recovered plasmid should contain the transgene with adjacent insertion site DNA, and this is highly advantageous as well for transposon-tagging and enhancer-trap screens, allowing easy isolation of the tagged genomic DNA. Drawbacks of this method are that it will add 2 to 3 kb of additional DNA to the vector, and, as mentioned above, antibiotic resistance genes may raise safety concerns for released transgenic insects.

Sequences adjacent to the insertion site should be different from those in the vector plasmid, and can be verified as such by alignment to the plasmid and using them to create primers for PCR. Genomic insertions can then be definitively verified by direct PCR of nontransformed host and transformed DNA, that should yield products of known length and sequence depending on the presence or absence of the transgene. It is also worthwhile to subject the chromosomal sequence to BLAST analysis (Altschul et al., 1990), since this will quickly determine if the sequence is nonplasmid (which is not always evident if it is rearranged), and may possibly identify interesting sequences within the genome by homology to sequences within the database (with the sequence thus transposon tagged).

1.5 PERSPECTIVES ON THE USE OF INSECT GENE TRANSFER

As noted in the beginning of this chapter, and as will become more apparent throughout this book, the genetic transformation of insects will allow enormous strides in further understanding the genetic and biochemical basis of insect biology, and will present many new and efficient strategies to control the population and behavior of beneficial and pest insects. One only needs to review the incredible advancement in the knowledge of basic model systems such as *D. melanogaster, Caenorhabditis elegans*, mice, and *Arabidopsis thaliana*, made possible in large part by the analysis of transgenics. The primary motivation, however, for creating transgenic insects is for applied purposes, and in recent years we have already seen the revolutionary influence of gene transfer technology on the commercial uses of plant and animal systems. A major difference in the use of transgenic insects, however, is that unlike other transgenic organisms most of the applications for insects will require their release into the environment where their future and that of their descendants will not be under direct regulation. The only exceptions to this will be the release of sterile transgenics, created to optimize biological control programs such as the sterile insect technique (Handler, 1992; see Chapter 17 by Robinson and Franz), or the release of conditional lethals that will die in response to changing environmental conditions (Fryxell and Miller, 1994). Several other strategies discussed in this book rely on the release of genetically modified insects that are not sterile or lethal, and, as the technology advances, we can expect new and more sophisticated strategies requiring the release of fertile transgenics. This raises important questions relating to the ecological and environmental impact of these new insect systems, and it is clearly in our interest to consider these issues carefully as we develop strategies for the use of transgenic insects, and the specific design of vectors to create these strains.

Biological risk assessment and current regulatory practices for transgenic arthropod transport and release are addressed in this book (see Chapter 19 by Hoy and Chapter 20 by Young et al.), and it is realized that individual consideration must be given to each insect species modified by specific recombinant DNA molecules carried within particular vectors. Some of the greatest concerns, however, may be minimized by careful analysis of vector and marker function and by the use of available technology to manipulate the vector postintegration. For example, a major consideration for risk assessment is vector stability and the potential for horizontal transmission of transgenes into nontarget organisms. Use of bipartite vector systems and testing with transient assays should generally ensure vector stability in specific target host organisms, but other organisms in nature may have the same transposon or a related cross-mobilizing system (see Sundararajan et al., 1999), and in some contexts the transgene may have the opportunity to move into the genome

of such organisms if they possess a functional transposase, or integrase for viral systems. A method to prevent such movement is to create "suicide vectors" that allow the deletion or rearrangement of vector sequences, such as terminal and subterminal sequences, postintegration to immobilize the transgene effectively. Mechanisms by which this is possible are recombination systems such as FLP/*FRT* (Golic and Lindquist, 1989) and Cre/*lox*P (Siegal and Hartl, 1996), which are discussed in Chapter 3 by Rong and Golic. The use of transgenic markers such the green fluorescent protein (Prasher et al., 1992), which should be functional in almost all organisms (Chalfie et al., 1994; see Chapter 5 by Higgs and Lewis), also provides a means to effectively monitor released transgenics, as well as possible transgene movement outside the host species under experimental and field conditions.

From a more practical standpoint, one of major attendant problems with the ability to create transgenic strains is the required funds and facilities to rear them. A critical component in the use of transgenic organisms is to have many varied strains, some of which can be interbred for desired offspring, and it is not always obvious if or when a strain may be critical for a specific purpose. *Drosophila* was chosen as an organism for genetic research because its rearing was simple and took relatively little space (for a eucaryote at least), although at this point, where many thousands of wild, mutant, and transgenic strains exist, stock collections are routinely culled due to space and cost considerations. Almost all other insect systems are more difficult and costly to rear, and this has been a clear constraint on their use for genetic studies. Indeed, many important insect strains have been discarded due to lack of funds or the loss of a primary investigator. It is clear that for transgenic technology and genetic manipulation to advance for any insect species, the ability to store germ plasm must be a high priority. Possibilities include cryopreservation of insect embryos by chemical and mechanical means, which is already possible for some dipteran species (Leopold et al., 1998), as well as the potential for cold storage enhanced by expression of antifreeze proteins (AFP) that are produced by some insects for overwintering (Tyshenko et al., 1997). For the latter possibility, a marked chromosome with an appropriate AFP construct could be crossed into a strain for storage, and crossed out when necessary for normal rearing.

The creation of transgenic insects will provide many challenges for their safe, efficient, and effective use in laboratory and field studies, with vector stability and strain maintenance among them. It is our hope that the same thoughtfullness and creativity that is making this technology a reality will be used to meet these challenges effectively. Only in this way will the continued and productive use of transgenic insects go forward, allowing us to learn more about the biology of insects, as well as the ability to control their population size and behavior.

1.6 APPENDIX*

Equipment

1. Inverted or stereozoom microscope
   - — Magnification range of ~8 to 80×
   - — Transmitted and/or direct illumination
   - — Mechanical stage (Olympus BH2-SH with SZH-STAD1 adaptor can be mounted on most stereozoom microscopes)
2. Micromanipulators
   - — Narishige MN-151 (MN-152 or MN-153)
   - — Leica
   - — Eppendorf Injectman
3. Air-pulse injection systems

* Mention of a proprietary product does not constitute an endorsement or the recommendation for its use by USDA.

a. Clippard components (assembly needed)
   — Vinyl hose tubing, EV3-3 electronic valve, MAR-1 air regulator, PG-100 pressure gauge, MAF-1 air-filter (connect to air supply), L-fittings, T-fittings, hose-fittings, and gaskets
   — Male Luer fitting or cutoff 1-ml syringe (connect to hose fitting and needle holder)
   — Two 1.5-V C batteries and battery holder
   — Switch
   — WPI MPH-1 needle holder
   — House or tank air or nitrogen supply
b. WPI pneumatic picopump with foot switch
c. Eppendorf Transjector 5246

4. Needle preparation
   — WPI capillaries or needles
   — Drummond microcapillaries — 25 and 100 µl
   — Narishige needle puller
   — WPI needle puller
   — Sutter Instruments microbeveler BV-10
   — WPI micobeveler
   — Sigmacote
5. Egg dechorionation
   — Liquid bleach
   — Triton-X 100 or NP-40 nonionic detergents
   — 45-mm I.D. Buchner funnel
   — 500-ml Erlenmeyer flask
   — 43-mm-diameter white and black filter circles
   — Small vacuum chamber with drierite
   — Billups–Rothenberg tissue culture chamber MIC-101
   — Oxygen supply

Addresses

Billups–Rothenberg, Inc., P.O.Box 977, Del Mar, CA 92014

Bio-Rad Laboratories, Life Sciences Group, 2000 Alfred Noble Drive, Hercules, CA 94547-1804; (800) 4BIORAD; *www.bio-rad.com*

Clippard Instrument Laboratory, Inc., 7390 Colerain Rd., Cincinnati, OH 45239; (513) 521-4261; *www.clippard.com*

Eppendorf Scientific, Inc., Cantiague Lane, Westbury, NY 11590-2852; (516) 876-6800; *www.eppendorfsi.com*

Leica Microsystems, Inc., 111 Deer Lake Rd., Deerfield, IL 60015-4986; (847) 405-0123; *www. leica-microsystems.com*

Narishige USA, Inc., 1 Plaza Rd., Greenvale, NY 11548-1027; (516) 621-4588; *www.narishige.co.jp/main.htm*

Olympus America, Inc., 2 Corporate Center Drive, Melville, NY 11747-3157; *www.olympus.com*

Qiagen, Inc., 28159 Stanford Ave., Valencia, CA 91355; (800) 362-7737; *www.qiagen.com*

Sigma Chemical Co., 3050 Spruce St., St. Louis, MO 63103-2564; (800) 325-3010; *www.sigma-aldrich.com*

Sutter Instruments Co., 51 Digital Drive. Novato, CA 94949; (415) 883-0128; *www.sutter.com*

World Precision Instruments Inc., 175 Sarasota Center Blvd., Sarasota, FL 34240-8750; (941) 371-1003; *www.wpiinc.com*

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